Fluorescence overlay antigen mapping of the epodermal basement membrane zone with reference to the different diagnosis of bullous pemphogoid and epidermolysis bullosa acquisita

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7

Summary

Immunofluorescence microscopy (IFM) has become a standard tool for the diagnosis of a group of chronic "autoimmune" blistering diseases of the skin. This holds true in particular for the subepidermal immunobullous dermatoses which are characterized by (a) the development of vesicles and blisters clinically, (b) a subepidermal split histologically, and (c) a characteristic fluorescence staining pattern of immune reactants at the epidermal basement membrane zone (EBMZ). Among the subepidermal immunobullous dermatoses, bullous pemphigoid (BP) and epidermolysis bullosa acquisita (EBA) commonly present difficulties in differential diagnosis because of overlapping features of clinical, histological and standard immunofluorescence findings. The latter include a homogeneous linear fluorescence staining pattern of *in vivo* bound IgG and/or C3c at the EBMZ. In both diseases this characteristic, though indistinctive pattern has become a major diagnostic hallmark at the level of the light microscope. Immunoelectron microscopic (IEM) studies have shown that in BP and EBA the *in vivo* bound IgG can be detected at different, disease-specific topographic sites of the EBMZ. The EBMZ is composed of four distinct ultrastructural areas: (a) the plasma membrane at the dermal site of the epidermal basal cells containing hemidesmosome complexes, (b) the lamina lucida, an electron lucent area, (c) the lamina densa, and (d) the sublamina densa area which includes anchoring fibrils, dermal microfibrillar bundles and collagen fibers. In BP, the immune deposits are detected in the lamina lucida at the site of the hemidesmosome complex, while in EBA they reside either in the lower lamina densa or in the immediate sublamina densa area. Consequently, definitive diagnosis of BP and EBA can be accomplished by the ultrastructural identification of the site of skin-bound IgG deposits at the EBMZ. This test has become the golden standard for the differential diagnosis of BP and EBA. However, IEM is an expensive, technically demanding technique and therefore not widely available. The purpose of this study is to develop a light microscopic method for the differential diagnosis of BP and EBA using the immunofluorescence technique. This means that the following question has to be answered: Is it possible to develop a technique - using the fluorescence microscope - that permits discrimination between antigenic determinants that lie in close proximity topographically at the resolution limit of the light microscope? As described in this thesis, this question has eventually lead to the development of a modified immunofluorescence technique which I propose to call Fluorescence Overlay Antigen Mapping (FOAM). The FOAM technique combines multicolor immunofluorescence

methodology with overlay imaging by either photomicrography or videomicroscopy. The basic idea behind the development of this technique for the differentiation of BP and EBA is as follows: the unknown distribution of antigens that are in close proximity (IgG, either at the level of the lamina lucida, or at the level of the sublamina densa) may be located relative to a suitable antigen that is used as a topographic reference marker (TRM). The IgG with unknown topographic distribution, and the TRM with known topographic distribution are labeled in an immunofluorescence double staining procedure in which the antigens are tagged with differently colored fluorophores. Assessment of the distribution of IgG and the TRM relative to one another is done by visual inspection of an overlay image showing the distinctly labeled TRM and IgG. In the overlay image, similar antigenic distribution (overlap) is shown by blending of primary fluorescence colors (red, green and blue) into secondary colors of light (cyan, magenta, yellow and white). Reliable interpretation of these multicolor overlay images requires that the observer can unambiguously assign each color in the overlay images to the presence of a specific combination of the IgG and the TRM. In practice this means that some problems and pitfalls have to be addressed properly. First, geometric errors: the distinct red, green and blue signals ought to be positioned in exactly the same place in the overlay image, which is only approximately true in practice due to errors of the optical configuration. Therefore, the geometric fidelity of the overlay images has to be evaluated and corrected. Second, color fidelity: reliable interpretation of multicolor overlay images requires due consideration of the imaging errors that may compromize proper reproduction of the fluorescence colors in the overlay image. Third, resolution: is the resolution of the FOAM technique sufficient to discriminate between the different location of the IgG deposits in BP and EBA?
Since this thesis has been written from the point of view of the practitioner in the field of fluorescence microscopy, practical procedures are described for assessment and correction of the above-mentioned technical conditions that are required to ensure faithful performance of the FOAM technique. They should be of help to enable reliable assessment of the topographic relation of antigens close to the resolution limit of the light microscope.

In the introduction (**Chapter one**), a brief overview is presented of the main clinical, histopathological, immunofluorescence and ultrastructural aspects of BP and EBA. It is explained why these aspects of the two diseases may give rise to diagnostic problems. Furthermore, FOAM is introduced as a light microscopic diagnostic and research technique that might be used for the differential diagnosis of the two diseases. It is shown that reliable use of FOAM procedures requires special attention given to some problems and pitfalls. These are studied in the respective chapters of this thesis.

In the first study (**Chapter two**), geometric errors are studied in skin tissue sections. Procedures are described for assessment and manual correction of the major geometric error, the image shift difference. Furthermore, an alignment verification test is described to assess the accuracy of the image shift correction procedure.

In the second study (**Chapter three**), practical definitions of errors that may impair color fidelity are provided, with special reference to photomicrographic overlaying. Attention is given to (a) spectral matching errors, (b) image contrast errors, and (c) exposure time errors. The errors studied, using immunofluorescence staining of EBMZ antigens as a performance test model, enables the user to formulate technical conditions that are required for faithful interpretation of the primary and secondary colors in overlay images.

The third study (**Chapter four**), on the FOAM technique addresses the question which differences of antigen distributions close to the resolving power of the light microscope can be distinguished. For this purpose a topographic staining model in human skin was developed, using structural EBMZ antigens as topographic reference markers. The distribution of these markers relative to one another is visualized by the videomicroscopic variant of the FOAM technique. In this study the technical conditions concerning geometric- and color fidelity (determined in the preceding two chapters) were incorporated in the FOAM procedures to ensure reliable interpretation of the relative antigen distributions in the overlay images. The results show that it is possible to distinguish topographic differences of antigen distributions with an upper resolution limit of 200±50 nm. Furthermore, the findings indicate that collagen Type VII and β4 integrin are the most suitable molecules to serve as topographic reference marker in the diagnostic application of the technique aiming at the differentiation of BP and EBA.

The fourth study (**Chapter five**), shows the "proof of the pudding". The differentiation of BP and EBA by the developed FOAM technique was tested on skin samples of patients with established diagnose. The technique was applied to perilesional skin from 7 patients with BP and 6 with EBA, using computer-aided overlay imaging of red-stained type VII collagen and green-stained IgG according to findings in the preceding chapter. It was shown that the FOAM technique allows for differentiation indeed between IgG deposits above (BP) and just below (EBA) the lamina densa of the EBMZ.

In the last chapter (**Chapter six**), the results obtained so far are summarized and integrated. Some future perspectives regarding the diagnosis of bullous dermatoses and application of the FOAM technique in biomedical research are discussed. It is concluded that FOAM may greatly enhance the potential of fluorescence microscopy when due consideration is given to a number of pitfalls that are prone to obstruct the geometric- and color fidelity of the overlay images. The evaluation and correction procedures provided may serve as a solid starting point for successful use of the FOAM technique in many fields of clinical diagnosis and biological research.